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Production of infectious poliovirus from cloned cDNA is dramatically increased by SV40 transcription and replication signals

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ABSTRACT

Sub-genomic cDNA clones representing the entire genomic RNA of poliovirus Type 1 (Mahoney) have been isolated in E. coli. Construction of a complete cDNA copy of the poliovirus genome in the EcoRI site of plasmid vector pBR325 from these clones is described. Introduction of plasmid DNA containing the complete cDNA copy of polio RNA into cultured primate cells by transfection produces infectious poliovirus. The virus produced by such a transfection appears to be identical to wild type poliovirus. Isolation of a polio recombinant plasmid containing SV40 transcription and replication signals is also described. Transfection of COS-1 cells with this plasmid yields greater than 1,600 plaque-forming units (PFU) per μg of input DNA.

INTRODUCTION

The molecular biology of poliovirus, a member of the picornavirus group, has been well-characterized with respect to details of the virus life cycle and the primary structure and genetic organization of the viral RNA. The complete nucleotide sequence of the nearly 7.5 kb plus strand genomic RNA that is 3'-polyadenylated and 5'-linked to a small polypeptide (VPg) was reported more than 2 years ago (1,2). Further structural studies based on this sequence elucidated the precise genomic encoding locations of nearly 20 viral-specific polypeptides as well as the amino acid pairs cleaved in the giant polyprotein precursor (the only translation product of poliovirus mRNA) to generate these polypeptides (3,4,5,6,7). Yet, to date, the functions of only two nonstructural polypeptides specified by the viral RNA have been elucidated. Protein P3-4b (also called p63) has been shown to be a primer-dependent viral RNA polymerase (8,9). Protein P3-7c is the viral proteinase that cleaves at nearly all of the glutamine-glycine pairs used in the processing of most precursor polypeptides (10). The functions of the majority of the viral-specific proteins found in infected cells (e.g., P2-3b, P2-5b, P3-2, VPg) are largely unknown. In order to determine the functions of these nonstructural polio proteins, we have initiated experiments to genetically manipulate the poliovirus genome. To this end we and others (2,11,12,13) have generated cDNA copies of poliovirus RNA and have cloned these copies in E. coli.

In this paper, we describe the isolation of three sub-genomic cDNA clones of

poliovirus RNA that, together, represent the entire viral genome. One of these clones, pAD337, contains the 5' proximal sequences of polio RNA, including the 5' canonical nucleotides. A second clone, pKE195, contains the 3' terminal sequences of the poliovirus genome, including a poly(A) tract of 84 adenylate residues. The third clone, pKE88, has sequences originating from the middle region of the viral RNA. The above three clones were used to construct a complete copy of the poliovirus genome in the EcoRI site of the plasmid vector pBR325. We show that when DNA from a plasmid containing the full-length cDNA copy of poliovirus RNA (pDS303) is introduced into cultured primate cells, infectious poliovirus is produced. Our results are thus in agreement with those published previously by Racaniello and Baltimore (14). In addition, we show that simian virus 40 (SV40) transcription and/or replication signals increase the production of poliovirus from cloned plasmid DNAs by 18- to 250-fold, depending upon the cell line used for transfection.

MATERIALS AND METHODS

Bacterial Strains

Routine propagation of plasmids was carried out in E. coli HB101. Transformation with plasmid DNA was carried out in E. coli HB101 or JA221 lpp^-/F' $lacI^q$. The latter strain is an E. coli K-12 derivative (15) that takes up DNA very efficiently (K. Nakamura and M. Inouye, personal communication).

Synthesis of Poliovirus Complementary DNA (cDNA)

Synthesis of single strand cDNA was carried out with purified poliovirus RNA, oligo $dT_{(12-18)}$, and avian myoblastosis virus reverse transcriptase (Life Sciences, Inc) as previously described (16,17). We also included 500 units/ml human placental RNase inhibitor (RNAsin; Biotech) in our cDNA synthesis reactions. After synthesis at 37°C or 42°C for 2 hr, the reaction mixture was phenol:chloroform (1:1) extracted and the nucleic acids were recovered by ethanol precipitation. The sample was then dissolved in 250 µl of 20 mM EDTA, 0.1 N NaOH and incubated for 1 hr at 60°C. The reaction mixture was neutralized with HCl, chromatographed on a small (0.5 cm x 10 cm) Sephadex G-200 column to separate the polio cDNA from free nucleotides, and the purified cDNA was recovered by ethanol precipitation.

Second Strand Synthesis and Tailing Reactions for 5' cDNA Clones

Approximately 2 µg of cDNA (from above) was tailed with dCMP at the 3' end using terminal deoxynucleotidyl transferase (P. L. Biochemicals) as described by Deng and Wu (18). After incubation for 20 min at 30°C, the reaction mixture was extracted with phenol:chloroform (1:1) and precipitated with ethanol. The resulting pellet was resuspended in 76 mM TRIS-HCl, pH 8.3, 90 mM KCl, and 23 µg/ml oligo $(dG)_{12-18}$ (Collaborative Research). Hybridization was at 56°C for 5 min and 50°C for 10 min

followed by chilling the reaction on ice (19). The reaction mixture was then adjusted to 120 mM KCl, 50 mM TRIS-HCl, pH 8.3, 10 mM $MgCl_2$, 10 mM DTT, 1 mM each of the 4 deoxynucleoside triphosphates, and 600 units/ml of reverse transcriptase and incubated for 90 min at 42°C. After phenol:chloroform extraction (1:1) and ethanol precipitation, the pellet was resuspended in 200 µl of 100 mM Hepes-KOH, pH 6.9, 10 mM $MgCl_2$, 10 mM DTT, 70 mM KCl, 0.6 mM each of the 4 deoxynucleoside triphosphates, and 70 units/ml Klenow fragment of DNA polymerase I (New England Biolabs). After incubation for 3 hr at 12°C, the reaction mixture was extracted with phenol:chloroform (1:1) and then ethanol precipitated. The double-stranded DNA (dsDNA) product was further purified by Sephadex G200 chromatography and then tailed at the 3' ends with dCMP as described above for single-stranded DNA.

Second Strand Synthesis and Linker Ligation Reactions for 3' cDNA Clones

Second strand synthesis to enrich for 3' genomic clones of poliovirus was accomplished using a synthetic primer [d(TACTACTGCG); Collaborative Research] that corresponds to nucleotides 3569-3578 on the poliovirus genome (1). Approximately 4 µg of single-stranded poliovirus cDNA (synthesized and purified as described above) was mixed with 10 µg of the synthetic oligonucleotide d(TACTACTGCG) in 70 µl of 5 mM TRIS, pH 8, 0.5 mM EDTA. The reaction mixture was heated at 90°C for 3 min and then quick-cooled on ice. After the addition of 5 µl of 3 M NaCl, the reaction mixture was incubated at 60°C for 30 min. The reaction mixture was then adjusted to 50 mM Hepes, pH 6.9, 5 mM $MgCl_2$, 5 mM DTT, 0.2 mM each of the 4 deoxynucleoside triphosphates, 250 units/ml Klenow fragment of DNA polymerase I (New England Nuclear) and incubated at 37°C for 90 min. The reaction was terminated by phenol:chloroform (1:1) extraction and the products recovered by ethanol precipitation. The dsDNA was further purified by Sephadex G-200 column chromatography and ethanol precipitation. The above DNA was then digested to completion with restriction endonuclease KpnI followed by phenol:chloroform (1:1) extraction and ethanol precipitation. The 3' protruding ends (a result of the KpnI digestion) were made blunt by incubating the digested, dsDNA with 2 units of the Klenow fragment of DNA polymerase I in 50 mM TRIS-HCl, pH 8, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol, 50 µg/ml bovine serum albumin, 20 µM EDTA, and 0.12 mM each of the 4 deoxynucleoside triphosphates. After incubation at 15°C for 60 min, the reaction mixture was phenol:chloroform extracted and ethanol precipitated. The blunt-ended DNA (from above) was ligated to synthetic EcoRI linkers (New England Biolabs) by incubation with 4.5 units T4 DNA ligase (Bethesda Research Laboratories) in 50 mM TRIS-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 1.0 mM ATP, and 50 µg/ml bovine serum albumin at 12°C for 12 hr. The linker-ligated DNA was then digested with EcoRI (to remove linker multimers) and purified by chromatography on a Sephadex G-200 column.

Annealing and Ligation Reactions

For cDNAs enriched for 5' polio sequences, 0.25 µg of dCMP-tailed ds cDNA was annealed to 0.5 µg of dGMP-tailed, PstI-digested pBR322 DNA in a 50 µl reaction containing 10 mM TRIS-HCl, pH 8.3, 0.15 M NaCl, and 1 mM EDTA. The mixture was incubated at 55°C for 10 min, 42°C for 2.5 hr, 20°C for 1 hr, and 4°C overnight. This mixture was then used directly for transformation of E. coli HB101. For cDNAs enriched for 3' polio sequences, approximately 0.3 µg of polio ds cDNA (containing EcoRI linkers) was incubated with 0.4 µg of EcoRI-digested, alkaline phosphatase-treated pBR325 DNA (20,21) and 3 units of T4 DNA ligase in 50 mM TRIS-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 1.0 mM ATP, and 50 µg/ml bovine serum albumin. Ligation was for 14 hr at 12°C and this mixture was used directly for transformation of E. coli JA221 lpp^-/F' $lacI^q$. All subsequent subcloning steps employed the same ligation conditions but these mixtures were used to transform E. coli HB101.

Transformation of E. coli and Screening of Plasmid Recombinants

Transformation of E. coli (made competent by $CaCl_2$ treatment) was carried out using slight modifications of published procedures (22,23). Transformants with pBR322 derivatives were screened for tetracycline resistance, ampicillin sensitivity and transformants with pBR325 derivatives were screened for ampicillin resistance, chloramphenicol sensitivity. Plasmid DNAs from individual colonies were analyzed by a slight modification of the rapid alkaline lysis procedure (24) and subsequent restriction endonuclease digestions.

DNA Purification and Sequencing

Plasmid DNAs were isolated by the method of Godson and Vapnek (25) as modified by J. Broach and colleagues (personal communication). Plasmid DNAs were purified by equilibrium centrifugation in cesium chloride-ethidium bromide gradients. Restriction fragments of DNA for subcloning experiments were purified by agarose gel electrophoresis followed by electroelution and DEAE Sephacel column chromatography as described (26). DNA sequencing was carried out according to the method of Maxam and Gilbert (27).

Transfection of Cultured Cells with Purified Plasmid DNA

Transfection of primate cells with polio-specific, recombinant plasmid DNAs was carried out using the calcium phosphate precipitation procedure (28). Transfections were carried out in duplicate on 80 - 90 % confluent monolayers of cells in 60 mm plates. For the transfections shown in Table 1, we included the glycerol boost step as previously described (29,30). The experiments shown in Table 1 were carried out using 5 - 10 µg of polio plasmid DNA per plate with either native salmon sperm DNA or pBR322 DNA as carrier (up to a total of 10 µg/plate). For the transfection data shown in Table 2, we omitted the glycerol boost step (to increase cell viability) and used 50 ng

to 5 µg of polio-specific plasmid DNA per plate. Native salmon sperm DNA (up to 10 µg/plate) was then added as carrier. After cultured cells had been incubated with the calcium phosphate-DNA precipitates for 4 hr at 37°C, a semi-solid overlay was added that contained 0.4% agarose (Seakem), modified Eagle medium, and 6% fetal calf serum. After 4 to 5 days at 37°, virus plaques were visualized by staining with either neutral red (for 293 cells) or crystal violet (for HeLa, CV-1, and COS-1 cells).

RESULTS

In order to obtain cDNA clones of both the 5' and 3' terminal sequences of poliovirus RNA, we employed two different cloning strategies. These strategies were independently optimized for the best representation of clones desired (i.e., either the 5' or 3' sequences of the polio genome).

Cloning of the 5' Half of Poliovirus RNA

To successfully clone the complete 5'-terminal sequences of poliovirus RNA, a method was used (19) that does not involve an S1 nuclease digestion step. Cloning RNA·cDNA hybrids, successfully used to produce clones of the entire poliovirus genome (11), was avoided in this study in order to decrease the risk of sequence rearrangements. This concern may actually be unwarranted (13). As outlined in Figure 1, poliovirus single-stranded cDNA was synthesized under optimum conditions to give predominantly full length cDNA copies of the genome (see Materials and Methods for details). Oligo (dC) tails were added to the 3' ends of this cDNA using terminal deoxynucleotidyl transferase and dCTP. Second strand synthesis was then primed by oligo$(dG)_{12-18}$ and accomplished by sequential incubation with avian myoblastosis virus reverse transcriptase and the Klenow fragment of DNA polymerase I. The double-stranded DNA was then tailed with dCMP at its 3' ends. Because second strand synthesis was not self-primed at the 3'-terminal hairpin, digestion with S1 nuclease to open up a terminal hairpin structure was not necessary.

The double-stranded DNA, synthesized and tailed as described above, was cloned into the dG-tailed PstI site of plasmid vector pBR322. Tetracycline-resistant, ampicillin-sensitive colonies of E. coli (HB101) were then screened for the presence of polio-specific inserts by rapid plasmid preparations and restriction endonuclease digestions. One of the clones obtained from the above transformation (which produced numerous clones containing polio-specific inserts from the entire genome) is shown at the bottom of Figure 1. This plasmid recombinant, called pAD337, contained polio sequences spanning from nucleotide 1 to ~4100 on the viral genome. The identification of the exact 5' end of poliovirus RNA within this recombinant plasmid was confirmed by DNA sequencing (31). In addition, the 5' viral sequences were preceded by 18 dG residues resulting from the tailing reactions prior to cloning. The 3' boundary of polio sequences

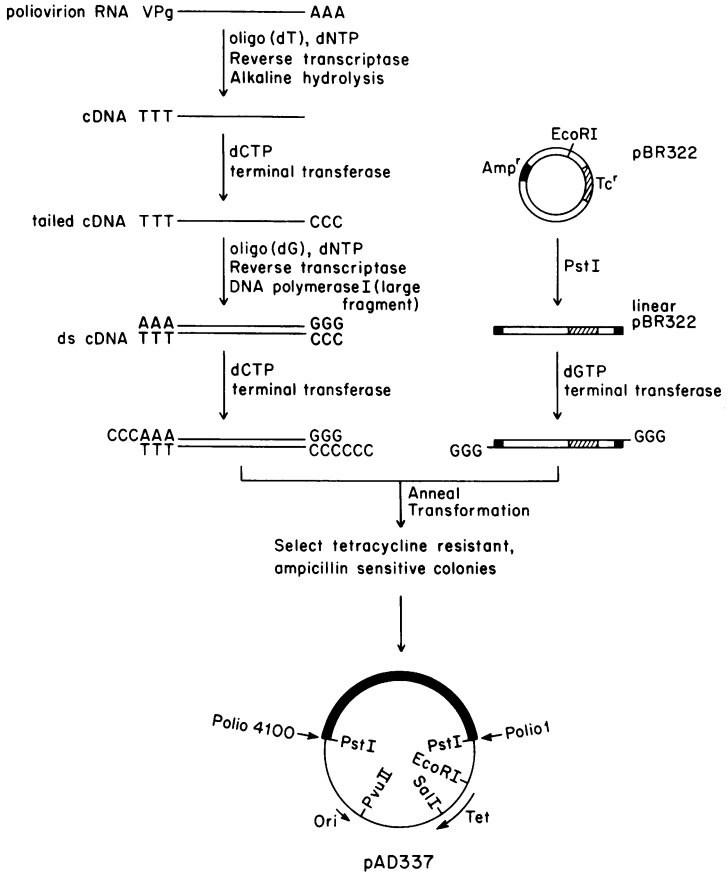

Figure 1. Strategy for isolation of recombinant plasmids of pBR322 containing 5' proximal poliovirus sequences.

within plasmid pAD337 was estimated to be at nucleotide 4100 by restriction endonuclease mapping (data not shown).

Cloning of the 3' Half of Poliovirus RNA

The strategy for obtaining 3' specific polio cDNA clones is outlined in Figure 2. This strategy is based upon the observation that polio 3' clones are difficult to generate even though reverse transcription is initiated at the 3'-terminal poly (A) with oligo (dT) (11,12,13). The reason for this phenomenon is unknown but a similar observation has been noted in the molecular cloning of aphthovirus RNA (32). After cDNA synthesis with reverse transcriptase, a double-stranded DNA was synthesized with the Klenow fragment of DNA polymerase I which uses the synthetic oligonucleotide d(TACTACTGCG) as well as the hairpin at the 3' end of the cDNA as a primer for

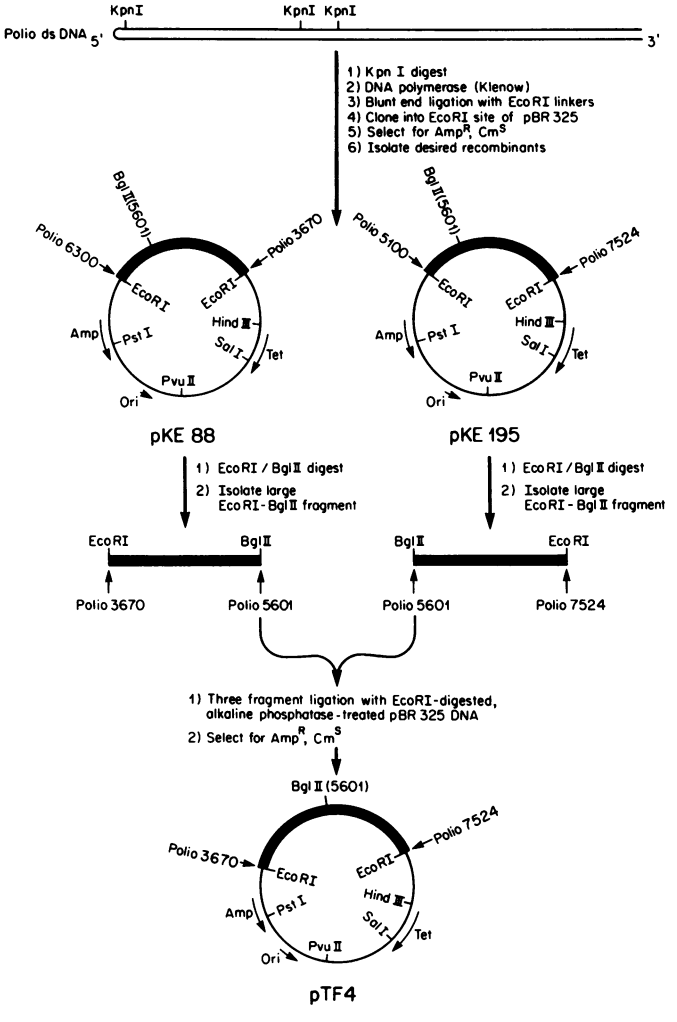

Figure 2. Strategy for isolation of recombinant plasmids of pBR325 containing 3' proximal poliovirus sequences.

synthesis. The dsDNA was then digested with restriction endonuclease KpnI, which cuts polio DNA at nucleotides Nos. 66, 3064, and 3660 (1). This step produces a desired DNA fragment beginning at nucleotide 3660 and ending (presumably) with the 3' poly(A) coding region. In addition, the above procedure has the advantage of not using S1 nuclease to nick the covalently-closed end of the dsDNA. S1 nuclease has the disadvantage of enzymatically removing nucleotides from the termini of duplex DNA. Such an activity could result in the shortening of the poly(A) tract at the 3' terminus of the ds

polio-specific DNA. After the KpnI digestion, the DNA fragments were treated with the Klenow enzyme to remove the 3' protruding tails produced by the KpnI digest, leaving blunt ends for linker ligation. The blunt-ended DNA was ligated to EcoRI linkers and cloned into the EcoRI site of plasmid vector pBR325 (20). Transformants in E. coli were selected for ampicillin-resistance and chloramphenicol-sensitivity. Rapid plasmid preparations and restriction digests were done on individual bacterial colonies to determine which clones contained 3' polio-specific inserts.

The above procedure did not yield an expected clone spanning the entire 3' half of the poliovirus genome. However, it did produce, among others, 2 clones containing 3' polio-specific DNA sequences. Plasmid pKE88 was shown by restriction mapping to contain polio nucleotides 3670 to 6300. Plasmid pKE195 contained a polio insert beginning with nucleotide 5100 and ending with the 3' poly(A) sequence. This latter fact was confirmed by Maxam-Gilbert DNA sequencing, which revealed a stretch of 84 adenylate residues directly adjacent to the 3' heteropolymeric sequence of the polio cDNA (B. Semler and L. Fox, unpublished results).

The two plasmids described above were used to construct a 3.8 kb polio insert contiguous to the 3' end. An EcoRI-BglII fragment (nucleotides 3670-5601) was joined to a BglII-EcoRI fragment (nucleotides 5601-7524) in a three fragment ligation with pBR325 DNA (digested with EcoRI). Amp^R, Cm^S transformants containing the desired insert (nucleotides 3670-7524) were recovered in both orientations within the pBR325 sequences. One of these plasmids (pTF4) was used in the construction of a full-length, infectious polio cDNA clone (see below).

Construction of a Full-Length cDNA Clone of Poliovirus RNA

The strategy employed to construct the full-length polio cDNA recombinant is shown in Figure 3 and Figure 4. This multi-step procedure was necessary because, in several instances, we were unable to obtain the necessary fragments after partial restriction enzyme digestion or derive clones containing the polio insert in the desired orientation (see below). Moreover, one of the overall goals of this cloning endeavor was to obtain a polio cDNA insert that could be easily moved to vectors containing transcription enhancer elements to increase expression of viral sequences. Such a "movable" insert could be obtained if synthetic EcoRI linkers were used, since there are no EcoRI sites in the polio genome (1,11). Indeed the recombinant plasmid pTF4 containing the 3' polio sequences was obtained using EcoRI linkers (refer to Figure 2). However, because we used a different method to clone the 5' end of polio RNA, the resulting insert was cloned into the PstI site of pBR322. Since there are three PstI sites in the polio genome (all located within the 5' 3500 nucleotides), it was necessary to modify the PstI site on the 5' side of polio nucleotide 1 to an EcoRI site. As outlined in Figure 3, plasmid pAD337 was digested to completion with restriction endonuclease PstI. The DNA

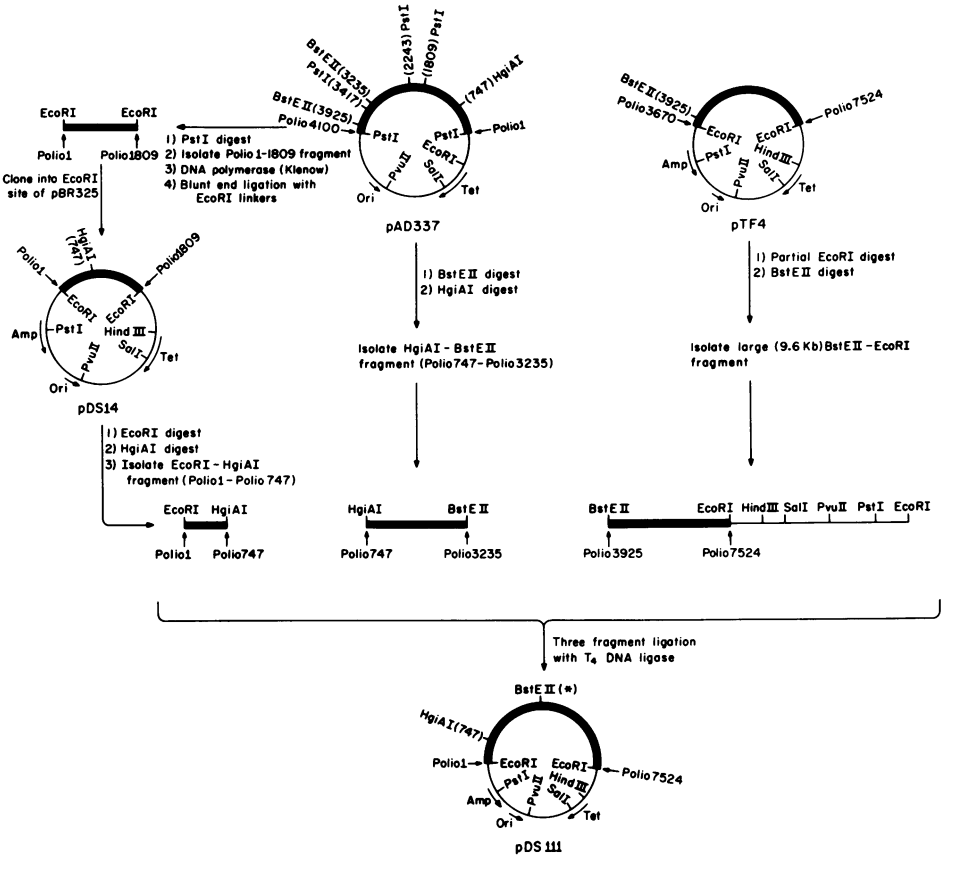

Figure 3. Strategy for construction of a recombinant plasmid containing a nearly full-length copy of the poliovirus. The asterisk at the BstEII site in pDS111 denotes the fact that this plasmid was generated without the 690 base pair BstEII fragment that spans nucleotides 3235-3925 in the polio genome.

fragment corresponding to polio nucleotide 1-1809 was isolated by agarose gel purification. The DNA fragment was then treated with the Klenow fragment of DNA polymerase I to produce blunt ends. The blunt-ended DNA fragment was incubated with synthetic EcoRI linkers and T4 DNA ligase and then cloned into the EcoRI site of plasmid vector pBR325. One of the resultant clones (pDS14) was digested to completion with EcoRI and HgiAI to produce the EcoRI to HgiAI fragment (polio 1 to polio 747) that constitutes the 5' fragment used in the three fragment ligation that produced pDS111 (Figure 3, see below).

The second DNA fragment used in the construction of plasmid pDS111 was ob-

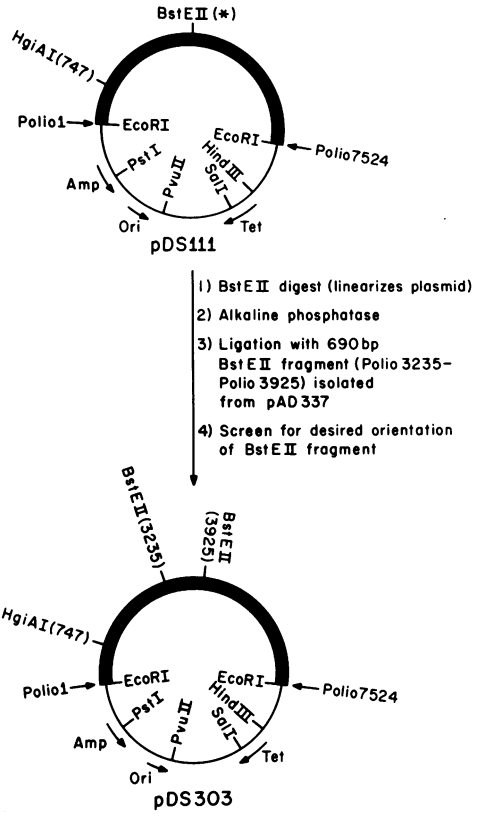

Figure 4. Final cloning step in the construction of the recombinant plasmid containing a complete cDNA copy of poliovirus RNA. Two other clones (pDS306 and pDS314) were also isolated from this step and they have an identical structure to pDS303.

tained by digesting pAD337 DNA to completion with BstEII and HgiAI. The resulting fragment (corresponding to polio nucleotides 747 to 3235) was then purified as described above. The third DNA fragment used to produce pDS111 originated from the 3' polio clone, pTF4. Plasmid DNA from pTF4 was partially digested with EcoRI in the presence of ethidium bromide (33). The mixture of partial fragments (after phenol/$CHCl_3$ extraction) was then digested to completion with BstEII. The desired 9.6 kb BstEII-EcoRI fragment was isolated and purified as described above. The three fragment ligation was then performed as shown in Figure 3. One of the ampicillin-resistant transformants of E. coli (following the above ligation reaction) contained the recombinant plasmid pDS111, shown at the bottom of Figure 3. The polio insert in this plasmid is a nearly full-length copy of the polio genome, lacking only the 690 bp BstEII

fragment (polio nucleotides 3235-3925) that was lost after the complete digestion of pAD337 with BstEII to generate the DNA fragment spanning polio nucleotides 747-3235.

It is worthy of note that we were unable to generate a partial digest of pAD337 with BstEII that would only cleave at site 3925, even in the presence of ethidium bromide. The reasons for no partial cleavage at this site are unclear but as a result, we were forced to do a complete BstEII digest of pAD337 and then re-insert the missing 690 bp fragment in a subsequent cloning step. As shown in Figure 4, the complete polio cDNA insert was constructed by digestion of pDS111 with BstEII and then ligation with the 690 bp BstEII fragment corresponding to polio nucleotides 3235 to 3925. After transformation, the resultant clones were screened for the desired orientation of the BstEII fragment by digestion with PstI, which cuts asymmetrically within the 690 bp BstEII fragment (at nucleotide 3417). Three clones (pDS303, pDS306, and pDS314) contained the BstEII fragment in the correct (i.e., polio "sense") orientation and thus contained a complete copy of the poliovirus genome. These findings were confirmed by extensive restriction mapping of the plasmid DNA isolated from pDS303, pDS306, and pDS314 (data not shown).

Production of Poliovirus by Transfection of Primate Cells with Cloned cDNA

To test whether our full length cDNA clone of poliovirus RNA would produce infectious virus upon introduction of plasmid DNA into primate cells, we carried out transfections of 293 cells using the modified calcium phosphate co-precipitation technique (28,29,30). We chose 293 cells (a human embryonic kidney cell line transformed with DNA from the early region of adenovirus; 34) because these cells efficiently take up and/or express foreign DNA introduced by transfection methods (J. Logan and T. Shenk, personal communication; see below). The results of the transfection experiments are summarized in Table 1. When 293 cells were transfected with plasmid DNA from pDS303, we obtained an average of 3 plaques per μg of DNA. In addition, plasmid DNA from pDS306, a second recombinant clone containing a full-length copy of the polio genome, yielded 0.9 plaques per μg. Five plaques from a pDS303 transfection and three plaques from the pDS306 transfection were picked and used to infect fresh monolayers of HeLa cells. All eight of the above plaques caused total cell death of the newly-infected monolayers within 48 hr post-infection, thus demonstrating that infectious virus had been produced by transfection with cloned cDNA.

The harvests from HeLa cell monolayers that had been infected with four of the plaques from the above transfection of 293 cells with pDS303 DNA were titered on HeLa cells. These virus titers ranged from 1.3×10^7 PFU/ml to 1.1×10^8 PFU/ml. In addition, we tested whether the virus obtained from infection of HeLa cells by the above four plaques could be neutralized by Mahoney-type specific monoclonal antisera to poliovirus. At least 6 logs of virus originating from each of the four plaques could be

Table 1. Yields of infectious poliovirus after transfection of primate cells in culture

Source of DNA	Cell Line	PFU/μg
1) pDS303	293	3.0*
2) pDS303 + RNase	293	1.3
3) pDS303 + BamHI	293	0
4) pDS303	HeLa	0.4*
5) pDS303	CV-1	0.6*
6) pBR322	293	0
7) Salmon sperm	293, CV-1	0
8) pDS306	293	0.9
9) pDS306	CV-1	0.4

* Average of three or more experiments

completely neutralized by two different monoclonal antibodies (35; Semler, Emini, and Wimmer, unpublished). We conclude that the virus produced by transfection with cloned poliovirus cDNA has not undergone any mutational event to alter its Type 1 (Mahoney) surface structure.

As shown in Table 1, treatment of pDS303 DNA with RNase prior to transfection did not abolish the infectivity of the cloned cDNA, although it did reduce the number of plaques obtained compared to that obtained with the untreated DNA. A similar reduction in virus yield after RNase treatment of cloned polio cDNA was observed by Racaniello and Baltimore (14). When plasmid DNA from clone pDS303 was digested with BamHI, an enzyme which cleaves within the polio sequence at 5 sites, no plaques were observed (refer to Table 1). The ability of plasmid DNA from pDS303 to produce infectious poliovirus is therefore due to the DNA itself and not to any contaminating poliovirus RNA.

We also carried out transfection experiments with our cloned polio cDNAs using CV-1 (monkey) cells and HeLa (human) cells. As shown in Table 1, the yields of virus obtained after transfection with either pDS303 or pDS306 DNA were reduced when compared to those obtained from 293 cell transfections. Subsequent experiments have consistently shown lower virus yields obtained from transfection of CV-1 or HeLa cells with cloned, infectious polio cDNA when compared to transfection of 293 cells with the same DNA. Since poliovirus normally grows to high titers in both CV-1 and HeLa cells,

the observed differences in virus yield must be due to better DNA uptake and/or expression of foreign DNA by 293 cells.

Increased Production of Poliovirus from Recombinant Plasmids Containing SV40 Sequences

The levels of specific infectivity obtained for polio recombinant plasmids in this and a previous study (14) were less than 10 plaque-forming units (PFU)/μg of plasmid DNA used in transfection experiments (refer to Table 1). Such low levels of virus produced from cloned DNA would make it impossible to differentiate between a non-viable mutant and a viable mutant that grows only 1% as well as the wild type virus generated by transfection. Since our cloned polio cDNAs will be used to assay viral functions after site-directed mutagenesis, we attempted to increase the specific infectivity of polio-specific cDNAs by constructing plasmids that contain the SV40 transcription signals and origin of replication. Figure 5 shows the construction of one such plasmid, pEV104. The plasmid vector pBR322 (36,37) was digested with restriction endonuclease AccI to remove a 1.6 kb DNA fragment containing a portion of the tetracycline resistance determinants as well as a sequence thought to be refractory toward SV40 DNA replication in simian cells (38). The resulting plasmid (pNT4) was digested with restriction endonuclease HindIII and ligated to a 1.1 kb HindIII fragment from the SV40 genome that contains (i) the promoters for both early and late transcription, (ii) the 72 base pair repeat sequences (shown to act as enhancers of viral [39,40] and non-viral [41] transcription), and (iii) the origin of replication. Transformants of E. coli from the above ligation were screened for orientation of the SV40 HindIII fragment and plasmid pES131 (shown in Figure 5) was chosen for insertion of the 7.5 kb polio insert from pDS303 (refer to Figure 4). A ligation reaction with EcoRI-digested pES131 and the 7.5 kb EcoRI polio-specific fragment from pDS303 and subsequent transformation of E. coli yielded transformants that contained, among others, the plasmid (pEV104) shown at the bottom of Figure 5.

To study the effects of the SV40 transcription and replication signals on the expression of polio-specific sequences, we used plasmid DNA from pEV104 in a series of transfection experiments of various primate cell lines. The results of these transfection experiments are shown in Table 2. The specific infectivity (PFU/μg) of pEV104 DNA is increased 250-fold over that of pDS303 when these DNAs are introduced into HeLa cells. A somewhat less dramatic but still significant increase is observed when CV-1 cells are transfected. An 18-fold increase in PFU/μg (compared to that of pDS303) was obtained when pEV104 DNA was used in transfections of CV-1 cells. As shown in Table 2, we also observed a 10-fold increase in specific infectivity when transfection of 293 cells with pEV104 DNA compared to transfection with pDS303 DNA. Note that the value of 27 PFU/μg for pDS303 transfection of293 cells is nearly

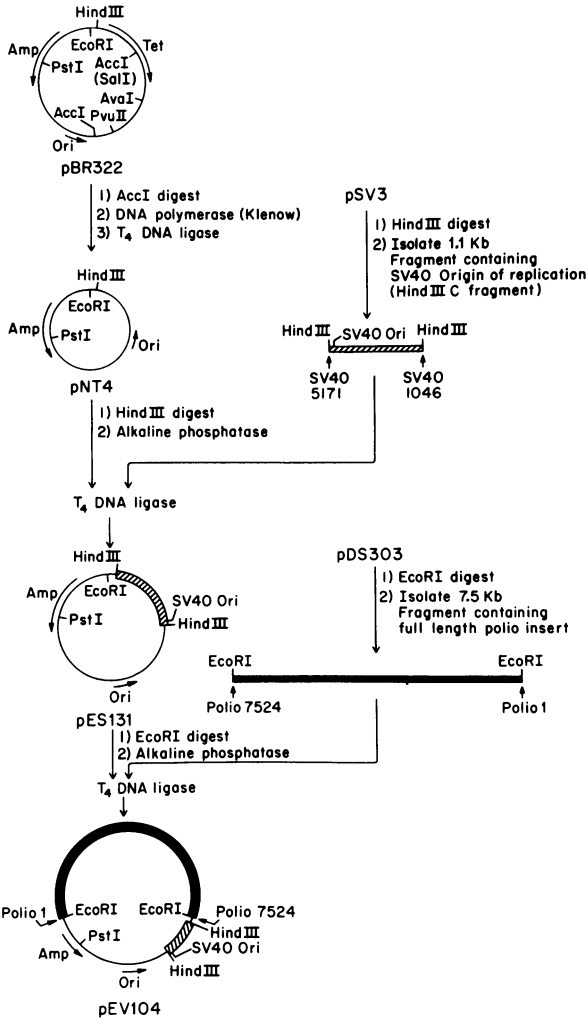

Figure 5. Scheme for construction of a polio recombinant plasmid containing SV40 transcription and replication regulatory sequences. Plasmid pSV3 is a clone of the SV40 genome inserted into the BamHI site of pBR322. The SV40 numbering system is taken from Buchman et al. (50).

10-fold higher than the corresponding number shown in Table 1. This increase is due in part to using lower concentrations of polio-specific plasmid DNAs in the transfections shown in Table 2 (refer to Materials and Methods) and in part to an improved overall transfection efficiency for these latter experiments.

The highest level of specific infectivity was obtained when we introduced pEV104

Table 2. Effects of SV40 transcription and replication signals on transfection with recombinant polio plasmids.

Plasmid DNA	Cell Line	PFU/μg*
pDS303	HeLa	0.4
pEV104	HeLa	101
pDS303	CV-1	0.6
pEV104	CV-1	11
pDS303	293	27
pEV104	293	281
pDS303	COS-1	11
pEV104	COS-1	1,645

* Average of four experiments

DNA into COS-1 cells. The COS-1 cell line is derived from CV-1 cells that had been transformed with an origin-minus mutant of SV40 (42). These cells constitutively express the large T antigen of SV40, a protein required for replication of SV40 DNA (43). The data in Table 2 show that transfection of pEV104 DNA into COS-1 cells produces greater than 1,600 PFU/μg, a 150-fold increase compared to the specific infectivity of pDS303 in COS-1 cells. The increase to such high levels of specific infectivity must be due, in part, to the replication of input pEV104 DNA by virtue of the SV40 origin of replication present on this plasmid. Our preliminary experiments using Southern blot hybridization have shown at least a 25-fold increase in copy number in COS-1 cells for polio recombinant plasmids containing the SV40 origin of replication compared to those without the SV40 origin (C. Williams and B. Semler, unpublished observations).

DISCUSSION

We have described the isolation of three sub-genomic clones of poliovirus cDNA that were then used to construct recombinant plasmids containing a full length copy of the poliovirus RNA genome. These plasmids (i.e., pDS303 and pDS306) also contain a 3' poly(A) tract of 84 residues immediately adjacent to the heteropolymers tract of the polio genome. Although the 3' poly(A) tract of RNA isolated from purified poliovirions is heterogeneous in length (44), it has been reported to have an average chain length of 60 residues (45). Thus our clones containing complete cDNA copies of the polio genome have 3' poly(A) tracts that are equivalent in length to those found on viral RNAs. Since it has been suggested that the 3' terminal poly(A) is required for the infectivity of polio

RNA (46), a poly(A) tract of 84 nucleotides may contribute to the infectivity of plasmid DNA from pDS303.

The full length cDNA clones (pDS303 and pDS306) of the poliovirus genome that we have constructed in pBR325 from sub-genomic clones produce infectious poliovirus after transfection of primate cells. These findings are in agreement with those previously published by Racaniello and Baltimore (14) using independently derived sub-genomic clones and a different plasmid vector (pBR322). The virus isolated from individual plaques that were produced by transfection with DNA from plasmid pDS303 is authentic poliovirus Type 1 (Mahoney). It is completely neutralized by type-specific monoclonal antibodies to poliovirus (35). In addition, we have made a virus stock (called No. 2-3) from a single plaque isolate produced after transfection of 293 cells with pDS303. The viral RNA isolated from purified virions of virus stock No. 2-3 shows an identical RNase T1 fingerprint pattern to that of the wild type Mahoney virus (unpublished observations). Finally, growth curves of virus isolated after transfection with infectious polio cDNA are similar to those obtained with laboratory stocks of virus (R. Kuhn and E. Wimmer, unpublished results). Thus the viral genome did not undergo any major mutational alterations during the various steps of cloning and sub-cloning in bacteria or during the subsequent transfection of plasmid DNAs into cultured primate cells.

One problem encountered in this study was the instability of various clones containing poliovirus-specific sequences upon propagation in E. coli. Also, some plasmid inserts could only be obtained in a single orientation (e.g., the poliovirus-specific sequences in plasmid pAD337, shown in Figure 1). Attempts to clone the polio insert from pAD337 in the opposite orientation resulted in extensive rearrangements or deletions of the insert (31). A clone containing the entire 3' portion of the poliovirus genome was shown by direct sequence analysis to carry a sequence duplication and insertion of 180 base pairs at polio nucleotide 5251 (B. Semler, L. Fox, and R. Hanecak, data not shown). In addition, the clones containing the entire poliovirus genome are not stable if stored as L broth-agar stabs at room temperature. We routinely store all of our polio clones in glycerol at -20°C or -70°C. The reason for the observed instability is unknown. We suspect that fortuitous transcription of poliovirus cDNA sequences and the subsequent expression of viral proteins may be deleterious to the bacterial host cell. This may lead to the selection of plasmids that have rearranged or deleted, either partially or completely, the polio-specific sequences.

Our transfection experiments demonstrated that, for cell lines that do not replicate polio-specific plasmid DNAs, higher specific infectivities were always obtained in 293 cells (refer to Table 1 and Table 2). The reason for the increased expression of polio sequences in transfected 293 cells may be the constitutive expression of the

adenovirus E1A gene in these cells (34). It has recently been shown (47,48) that the constitutive expression of the E1A gene in 293 cells causes increased expression of transfected plasmid DNAs containing either the adenovirus E2 gene or the human β-globin gene. The mechanism by which the adenovirus E1A gene product acts in trans to stimulate transcription of other genes is unknown. Perhaps the E1A protein acts as a transcriptional activator that induces the formation of stable transcriptional complexes, as suggested by Gaynor and Berk (49). Such an activity might be particularly important for expression of polio-specific sequences from pDS303, since this plasmid carries no authentic promoter-regulatory signals for RNA polymerase II transcription and presumably depends on "fortuitous" transcription initiation. Studies are in progress to determine how transfected poliovirus cDNA is transcribed in cells.

We have increased the expression of polio-specific sequences from plasmids introduced into primate cells by constructing recombinant vectors containing SV40 transcription/replication regulatory sequences. The presence of authentic RNA polymerase II promoters on plasmid pEV104 causes a significant increase in production of poliovirus after transfection when compared to pDS303 transfections. Presumably, the promoter for early SV40 transcription (located near the origin of replication) functions to direct transcription through plasmid DNA sequences and then into polio-specific sequences (corresponding to transcription in a clockwise direction around pEV104; refer to Figure 5). Most or all of the increase in specific infectivity of pEV104 in the HeLa, CV-1, and 293 cell lines must be due to increased transcriptional activity because these cells lack the large T antigen function required to initiate DNA replication using the SV40 origin sequences (43). However, when pEV104 DNA is introduced into COS-1 cells, plasmid copy number can be increased due to the constitutive expression of large T polypeptide in these cells. The high level of specific infectivity that we obtained for pEV104 transfection of COS-1 cells (1,645 PFU/μg; refer to Table 2) probably reflects an increase in plasmid copy number as well as an increase in polio-specific transcription.

The availability of plasmid DNAs that initiate a poliovirus infection after being introduced into primate cells in culture provides a basis for a molecular genetic approach not previously available for a functional analysis of the poliovirus RNA genome. Directed mutagenesis of specific regions of the poliovirus genome should answer important questions about the functions of many of the non-structural polypeptides coded by the viral RNA. In addition, generation of mutants in and around specific sites for protein processing (i.e., glutamine-glycine or tyrosine-glycine amino acid pairs) will provide information about critical determinants required for cleavage by the viral proteinase. The increased specific infectivities that we have obtained for

pEV104 transfection of COS-1 cells will allow a more sensitive detection and biochemical analysis of such mutants.

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